Reference: *Biol. Bull.* **166:** 473–481. (June, 1984)

SPECIFICITY IN THE INDUCTION OF POLYSPERMY IN SEA URCHIN EGGS BY SOYBEAN TRYPSIN INHIBITOR

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Abstract

Sea urchin egg cortical granules contain a soybean trypsin inhibitor (SBTI)-sensitive protease that is activated and secreted upon fertilization. Results obtained in several laboratories have implicated this activity in the cortical reaction, elevation of the fertilization envelope, and the establishment of the block to polyspermy. These conclusions are supported in part by studies using a variety of trypsin inhibitors. This interpretation has recently been challenged, however, and past results with soybean trypsin inhibitor (SBTI) and other agents were attributed to a non-specific protein effect (Dunham *et al.*, 1982). We have re-examined this question by studying the effects of native and inactivated SBTI on purified egg protease and the promotion of polyspermy. Protease was purified from unfertilized eggs by SBTI-affinity chromatography in the presence of benzamidine-HCl to minimize autodegradation. Inactivation of SBTI by acid or alkali treatment greatly diminished its ability to inhibit the egg protease or to cause polyspermy. We also observed a correlation between the relative potencies of five serine protease inhibitors and their ability to promote polyspermy. We conclude that induction of polyspermy by SBTI and other protease inhibitors is indeed due to inhibition of the egg protease.

Introduction

The prevention of polyspermy in sea urchins is a multi-faceted process that includes structural, electrical, and enzymatic components. The block is usually considered to occur in two phases; an early but transient one, and a later but permanent one. Several events have been implicated in the early phase, including a rapid, sodium-dependent depolarization of the egg plasma membrane (Jaffe, 1976; Schuel and Schuel, 1981), the release of hydrogen peroxide by fertilized eggs which inactivates sperm in the vicinity (Boldt *et al.*, 1981; Coburn *et al.*, 1981), and the possible production of arachidonic acid oxidation products (Moss *et al.*, 1983; Schuel *et al.*, 1984). The permanent block to polyspermy is mediated by the cortical granule secretion-mediated lifting of the fertilization envelope which provides a stable physical barrier to further sperm penetration (reviewed by Schuel, 1978, 1984).

Evidence linking proteolytic activity in sea urchin eggs to the prevention of polyspermy, particularly with respect to the lifting of the fertilization envelope, has emerged over the last thirty years (reviewed by: Runnstrom, 1966; Epel, 1978; Schuel, 1978, 1984). Protease activity was observed early on in both unfertilized and fertilized eggs (Lundblad, 1954). In addition, soybean trypsin inhibitor (SBTI) and other trypsin inhibitors were found to impair fertilization envelope elevation and to cause polyspermy (Hagstrom, 1956). Subsequent studies showed that a SBTI-sensitive protease is localized

Received 30 January 1984; accepted 26 March 1984.

in the cortical granules of unfertilized eggs (Schuel *et al.,* 1973; Decker and Kinsey, 1983; Kopf *et al.,* 1983) and is secreted during the cortical reaction at fertilization or upon parthenogenetic activation (Vacquier *et al.,* 1972a, b, 1973; Carroll and Epel, 1975a, b; Fodor *et al.,* 1975; Carroll, 1976; Schuel *et al.,* 1976b; Carroll *et al.,* 1982). A serine protease has been isolated from unfertilized sea urchin eggs and fertilization product by SBTI-affinity chromatography and has been characterized biochemically (Fodor *et al.,* 1975; Alliegro and Schuel, 1983a).

The biological functions of protease(s) in fertilization have been studied by using SBTI and other specific enzymatic inhibitors, and by the direct application of purified preparations of egg protease to fertilization cultures. The protease-dependent processes which may be related to the prevention of polyspermy include: discharge of cortical granules and detachment of the vitelline envelope from the egg plasma membrane (Hagstrom, 1956; Lonning, 1967; Vacquier *et al.,* 1972a; Longo and Schuel, 1973; Longo *et al.,* 1974; Schuel *et al.,* 1976a, b); generation of H_2O_2 by fertilized eggs (Boldt *et al.,* 1981; Coburn *et al.,* 1981); and the inactivation of sperm binding sites on the vitelline layer (Vacquier *et al.,* 1973; Carroll and Epel, 1975a). Polyspermy arises in SBTI-treated eggs by refertilization at specific sites where the vitelline layer fails to detach from the egg plasma membrane (Longo and Schuel, 1973; Longo *et al.,* 1974; Schuel *et al.,* 1976b). Carroll and Epel (1975a) proposed the involvement of two distinct SBTI-sensitive proteases in the block to polyspermy: vitelline delaminase, which cleaves bonds between the vitelline envelope and plasma membrane; and sperm receptor hydrolase, which digests sperm receptors on the surface of the vitelline layer.

This entire line of investigation has recently been challenged (Dunham *et al.,* 1982). These authors attributed the induction of polyspermy by SBTI to a non-specific protein effect, rather than the specific inhibition of egg proteases. Their conclusion was based on the observation that other proteins (catalase, superoxide dismutase, bovine serum albumin, and gelatin) which are apparently unrelated biochemically to SBTI also cause polyspermy in *Arbacia* eggs. This interpretation casts doubt upon past results using protease inhibitors and other agents as probes to study the mechanisms involved in polyspermy inhibition.

We have re-examined this question by studying the effects of native and inactivated SBTI on the promotion of polyspermy. If SBTI causes polyspermy by a non-specific protein effect as proposed by Dunham *et al.* (1982), then its capacity to do so would not be dependent upon its ability to inhibit the purified egg protease. This hypothesis was tested by taking advantage of the observation that acid or alkali treatment of SBTI removes its ability to inhibit bovine pancreatic trypsin (Kunitz, 1947). In addition, we have compared the effects of several other serine protease inhibitors on inhibition of the egg protease and the induction of polyspermy. A preliminary account of this study has been reported previously (Alliegro and Schuel, 1983b).

Materials and Methods

Handling of gametes

Arbacia punctulata were obtained from the Marine Resources Department at the Marine Biological Laboratory, Woods Hole, Massachusetts, and *Strongylocentrotus purpuratus* from Pacific Bio-marine, Venice, California. Gametes were shed by intracoelomic injection of 0.5 *M* KCl (Harvey, 1956). Eggs were rinsed 2–3 times in filtered sea water (FSW). Sperm were stored "dry" on ice until needed.

Polyspermy experiments

Polyspermy experiments with protease inhibitors were carried out with *A. punctulata* gametes. Thirty microliters of settled eggs were preincubated for two minutes in 1.0 ml of FSW containing the desired inhibitor and inseminated with 40 μl of a 2.4% v/v sperm suspension. Based on an estimate of 2×10^{10} sperm/ml in dry semen (Harvey, 1956), the final sperm concentration in these experiments was approximately 2×10^7/ml. Lifting of the fertilization envelope was the criterion for determining fertilization. Polyspermy was estimated by counting the percentage of multipolar divisions at first cleavage (Schuel *et al.*, 1973). The incidence of fertilization was at least 95% in all experiments. Eggs were incubated at ambient temperature (24°–26°C) with occasional agitation. One hundred eggs were scored in each culture, and each experiment was repeated three times.

Enzyme isolation and assay

Protease was isolated from extracts of unfertilized *S. purpuratus* eggs by SBTI-affinity chromatography according to Fodor *et al.* (1975). Benzamidine-HCl (5 m*M*) was included to minimize autodegradation of the enzyme (Baginski *et al.*, 1982). Protease activity was measured by continuously recording the change in absorbance at 253 nm using benzoyl-1-arginine ethyl ester (BAEE) as substrate (Schwert and Takenaka, 1955). Reactions were initiated by adding 50 μl of the affinity-purified preparation (approximately 160 units/ml) to a cuvette containing 0.5 m*M* BAEE in 0.2 *M* Tris buffer, pH 8, and 25 μl of the appropriate inhibitor. One unit of activity is defined as $\Delta Abs_{253\ nm}$ of .001/minute. The total reaction volume was 2.5 ml. Inhibitors used were: soybean trypsin inhibitors (SBTI), lima bean trypsin inhibitor, chicken ovomucoid, antipain, and leupeptin. Antipain and leupeptin were gifts of Dr. Walter Troll. Soybean trypsin inhibitor-agarose for affinity chromatography was obtained from Bethesda Research Labs, Maryland. All other reagents were purchased from Sigma Chemical Co., St. Louis, Missouri.

Inactivation of SBTI

SBTI was inactivated according to Kunitz (1947). Briefly, a 0.5% solution of SBTI in 2.5 m*M* HCl was incubated at 90°C for 90 minutes for acid inactivation. A 0.25% solution of SBTI in 0.1 *N* NaOH was incubated for two hours at 36°C for alkali inactivation. Samples of inactivated inhibitor were dialyzed into FSW overnight at room temperature, and then diluted for experiments.

Determination of ID_{50} and P_{50}

To determine the inhibitor concentration which reduced enzyme activity to 50% of control levels (ID_{50}) or produced 50% polyspermy (P_{50}), regression curves were fit to the data points obtained for each of the inhibitors, and the 50% point calculated from the equation of line (Sokal and Rohlf, 1969). Coefficients of regression ranged from 0.93 to 0.99. Each experiment was repeated three times.

Results

Consistent with previous observations (reviewed by Schuel, 1978, 1984), SBTI inhibited the purified egg protease (Fig. 1) and caused polyspermy (Fig. 2). In our hands the isolation procedure developed by Fodor *et al.* (1975) yielded a single serine

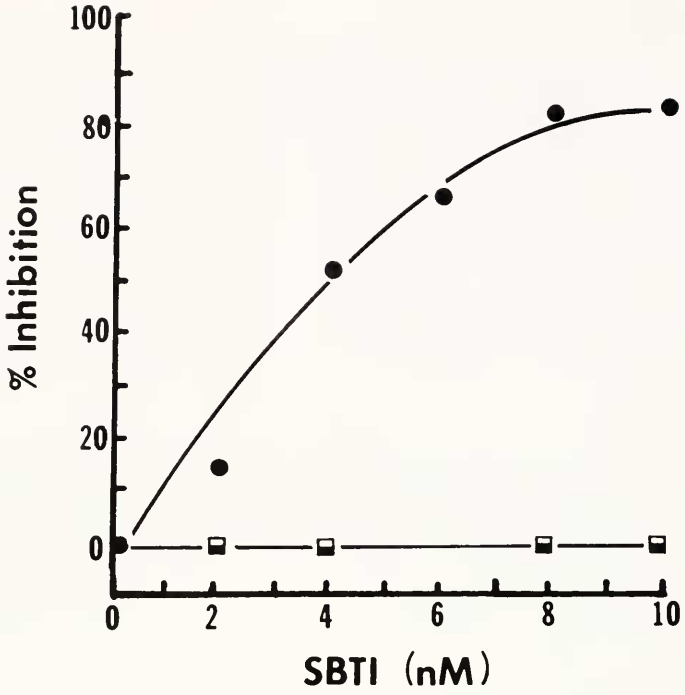

FIGURE 1. Effects of acid (□) or alkali (■) pretreatment on the ability of native (●) SBTI to inhibit protease activity isolated from unfertilized *Strongylocentrotus* eggs as described under Methods.

protease with a specific activity against BAEE comparable to bovine pancreatic trypsin (Alliegro and Schuel, 1983a). Acid or alkali inactivation of SBTI according to Kunitz (1947) greatly reduced its inhibition of the egg protease (Fig. 1) *and* its capacity to promote polyspermy (Fig. 2). These treatments did not result in 100% inactivation of SBTI since some inhibition of the egg protease activity was observed when the concentration of SBTI in the enzyme assays was increased 100-fold (data not shown). This residual native SBTI could account for the slight incidence of polyspermy observed in experiments with inactivated SBTI (Fig. 2). These results clearly demonstrate that the induction of polyspermy by SBTI depends upon its inhibition of the egg protease.

We also examined the effects of other serine protease inhibitors on the purified egg protease and the induction of polyspermy (Table I). In addition to SBTI, ovomucoid, lima bean trypsin inhibitor, leupeptin, and antipain were found to be effective inhibitors of the egg protease. The relative potency of these inhibitors against the egg protease is similar to that found for trypsins and related serine proteases isolated from a wide variety of sources (reviewed by Kassel, 1970; Umezawa, 1976). All five inhibitors of the purified egg protease promoted polyspermy. Each of the inhibitors severely impaired elevation of the fertilization envelope, which exhibited a rosette appearance as originally described by Hagstrom (1956). The data also suggest a correlation between the *relative* effectiveness of these compounds as protease inhibitors and polyspermy promotors; the stronger inhibitors promoted polyspermy at lower concentrations. According to Spearman's rank correlation (Sokal and Rohlf, 1969),

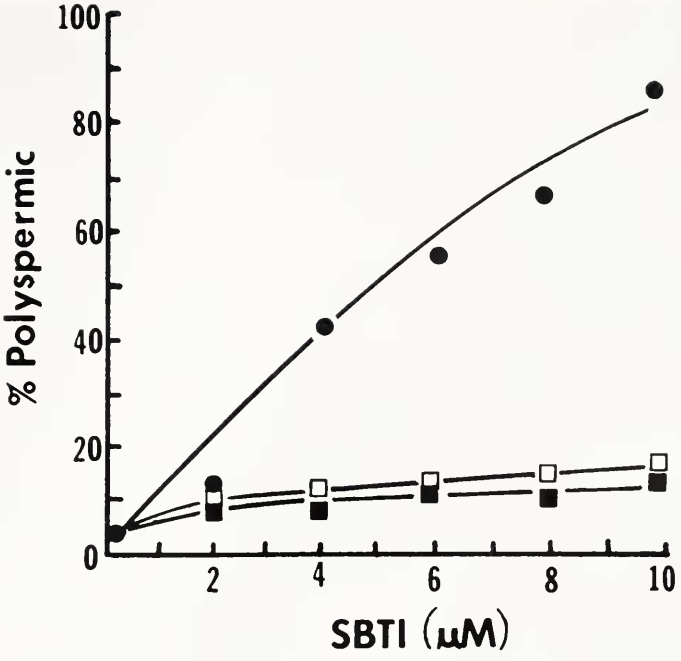

FIGURE 2. Effects of acid (□) or alkali (■) pretreatment on the ability of native (●) SBTI to cause polyspermy in *Arbacia* eggs as described under Methods.

r_s = 0.90. Thus, there is a statistical correlation between enzyme inhibition and polyspermy promotion by these serine protease inhibitors.

DISCUSSION

The question of specificity is a major concern in all studies involving the use of inhibitors. Past evidence for specificity in the induction of polyspermy by SBTI rested

TABLE I

Comparison of ID_{50} and P_{50} for inhibition of egg protease and promotion of polyspermy by serine protease inhibitors

Inhibitor	ID_{50} (*M*)	P_{50} (*M*)
Soybean trypsin inhibitor	6.01×10^{-9}	4.94×10^{-6}
Ovomucoid	1.06×10^{-6}	1.21×10^{-4}
Lima bean trypsin inhibitor	3.47×10^{-6}	1.42×10^{-4}
Leupeptin	4.22×10^{-6}	8.48×10^{-4}
Antipain	3.16×10^{-5}	3.69×10^{-4}

ID_{50} = the inhibitor concentration at which 50% of the enzyme activity purified from *S. purpuratus* eggs is abolished as described under Methods using BAEE as substrate.

P_{50} = the inhibitor concentration at which 50% polyspermy occurred in *A. punctulata* eggs, as described under Methods. The incidence of polyspermy for control eggs in these experiments was 3.3 + 0.58% (n = 15).

upon observations that it: inhibited the protease stored in cortical granules and secreted at fertilization; inhibited the serine protease purified from egg extracts or cortical granule exudates; and impaired fertilization envelope elevation. Furthermore, other serine protease inhibitors had similar effects (reviewed by: Runnstrom, 1966; Epel, 1978; Schuel, 1978, 1984). In fact, there appeared to be a relationship between their relative potencies as protease inhibitors and polyspermy promotors.

Nevertheless, Dunham *et al.* (1982) recently questioned the specificity of SBTI and other proteins as polyspermy promotors in sea urchins, and attributed their action to a non-specific protein effect. In our re-examination of this issue we found that acid or alkali inactivation of SBTI greatly diminishes its ability to inhibit the egg protease *and* to promote polyspermy. Thus, the protease inhibitory activity of SBTI is required for the protein to cause polyspermy. We have also confirmed previous observations that other serine protease inhibitors induce polyspermy in sea urchins (reviewed by Schuel, 1978, 1984). Furthermore, there is a correlation between their relative potencies as inhibitors of the egg protease and in their induction of polyspermy. Included in this group are antipain and leupeptin which are not proteins. Benzamidine and tosyl lysine chloromethyl ketone are other non-proteinaceous protease inhibitors known to induce polyspermy (Schuel *et al.*, 1973, 1976b). Based upon these results we conclude that the induction of polyspermy in sea urchins by protease inhibitors is due to a specific effect upon an egg protease.

Although the enzyme inhibition studies and polyspermy experiments were performed with different species, the correlation between the relative inhibitor potencies apparently holds true across species lines. These findings are consistent with previous observations using these inhibitors in *Arbacia, Strongylocentrotus,* and other species of sea urchins (Hagstrom, 1956; Vacquier *et al.*, 1972a, b; Schuel *et al.*, 1976a, b). This pattern emerges from the collective data in these earlier papers, but has not heretofore been presented in a single study performed under uniform conditions. That the correlation holds across species lines is not surprising, since a wide variety of trypsin-like proteases show a similar sensitivity profile with these inhibitors (Kassel, 1970; Umezawa, 1976). We did observe a difference in the absolute inhibitor concentrations required for the ID_{50} and P_{50} determinations. This is not surprising, considering the conditions of the two kinds of experiments. In the enzyme inhibition study, the inhibitor is competing with a synthetic substrate for a purified enzyme. In the biological experiment the inhibitor must compete effectively with natural substrates and under very different conditions than in the cuvette.

Dunham *et al.* (1982) reported that polyspermy can also be induced in sea urchins by catalase, superoxide dismutase, bovine serum albumin, and gelatin. These proteins are seemingly unrelated biochemically to SBTI or to each other. The authors hold this as evidence that the results obtained with these compounds are due to a non-specific protein effect. However, the chemical properties and existing evidence in support of specificity for each of these proteins must be carefully considered.

The function of catalase in aerobic systems is to remove and detoxify hydrogen peroxide (Chance, 1979). Sea urchin eggs produce H_2O_2 during fertilization (Foerder *et al.*, 1978; Boldt *et al.*, 1981). The fertilizing capacity of sea urchin sperm is significantly reduced by H_2O_2 (Evans, 1947; Boldt *et al.*, 1981; Coburn *et al.*, 1981). When catalase is added to fertilization cultures of *Arbacia,* the eggs become polyspermic (Coburn *et al.*, 1981; Dunham *et al.*, 1982). If the catalase is heat-denatured, the incidence of polyspermy is drastically reduced (Coburn *et al.*, 1981). Furthermore, catalase-induced polyspermy can be titrated away by the inclusion of 3-amino-1,2,4-triazole (Coburn *et al.*, 1981). Aminotriazole inhibits catalase by binding irreversibly

to the complex formed between the enzyme and its substrate, H_2O_2 (Margoliash *et al.*, 1960; Chance *et al.*, 1979). These observations provide strong evidence that catalase induces polyspermy by the specific removal of H_2O_2.

The induction of polyspermy by superoxide dismutase reported by Dunham *et al.* (1982) is not an altogether unexpected finding in view of the facts that superoxide anion is produced along with H_2O_2 during the respiratory burst of somatic cells, that it is itself cytotoxic, and that it can react with H_2O_2 via the Haber-Weiss reaction to form other extremely cytotoxic oxygen radicals (Klebanoff, 1980). Since the addition of catalase and superoxide dismutase can prevent the production of toxic oxygen radicals by phagocytic cells (reviewed by Chance *et al.*, 1979; Klebanoff, 1980; Haliwell, 1982), it is possible that similar reactions may account for the promotion of polyspermy by these enzymes in sea urchins. If this were true, then the actions of these two proteins on sea urchin gametes would in fact be functionally interrelated.

Gelatin is a classic substrate used in the assay of proteases (Northrup and Hussey, 1923; Northrup, 1930). It was one of the substrates used in early studies of sea urchin egg proteases (Lundblad, 1954). *A priori,* one might expect that gelatin could cause polyspermy by acting as a competitive substrate for egg proteases. The low levels of polyspermy observed by Dunham *et al.* (1982) are consistent with this idea. Finally, bovine serum albumin did not cause polyspermy in our hands (Coburn *et al.*, 1981; Schuel and Schuel, 1981; Schuel, 1984). However, it is also a potential protease substrate, especially if denatured.

We believe that limited proteolysis, the respiratory burst, the possible production of arachidonic acid oxidation products, and other early responses in gamete interaction are intimately related events, as they are in somatic cells (Schuel, 1984). Under these circumstances, it is possible that the "multiple mechanisms" implicated in the polyspermy block are in fact related as steps in one or a few associated metabolic pathways. The variety of agents capable of promoting polyspermy may be acting at different points in a sequence of reactions, perhaps interrupting the generation of some particular product(s) critical to polyspermy prevention.

ACKNOWLEDGMENTS

We would like to thank Drs. Walter Troll, Morris Zimmerman, and Jeffrey Boldt for reading this manuscript, and for their useful suggestions. Supported by NSF grant # PCM-82-01561 to H.S.

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